Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for *in vitro* Potency Testing of *Leptospira interrogans* Serovar *icterohaemorrhagiae* Bacterins

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1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) uses a sandwich enzyme-linked immunosorbent assay (ELISA) to measure the relative potency of bacterins containing *Leptospira interrogans* serogroup *icterohaemorrhagiae* (except strain BogVere) compared to a suitably qualified, nonexpired reference bacterin.

1.2 Keywords

Leptospira icterohaemorrhagiae, ELISA, potency, *in vitro*

2. Materials

2.1 Equipment/instrumentation

2.1.1 Micropipettors, to cover the range of 5.0 µl to 1000 µl

2.1.2 8- or 12-channel micropipettor, to cover the range of 50 µl to 200 µl

2.1.3 Orbital shaker

2.1.4 Automatic microplate washer (optional)

2.1.5 Microplate reader with dual wavelengths (405 nm and 490 nm)

2.1.6 Balance, validated from 150 mg to 15 g

2.1.7 Relative Potency Calculation Software (United States Department of Agriculture [USDA], Veterinary Services, Center for Veterinary Biologics-Laboratory [CVB-L]), current version

2.2 Reagents/supplies

2.2.1 96-well flat-bottom microtitration plates (Immulon 2, Dynatech Laboratories, Inc., or equivalent)

2.2.2 96-well microtitration plates suitable for making serial dilutions (transfer plate)

2.2.3 Plate sealers

2.2.4 Carbonate coating buffer

2.2.5 Phosphate buffer for elution (optional)

2.2.6 Sodium citrate for elution (optional)

2.2.7 Sodium desoxycholate elution buffer (optional)

2.2.8 Phosphate-buffered saline with 0.05% Tween 20 (PBS-Tween 20)

2.2.9 Antibody diluent

2.2.10 ABTS (2,2′-azino-di-3-ethylbenziazoline sulfonate) substrate, 1- or 2-component (Kirkegaard and Perry Laboratories, Inc., or equivalent)

2.2.11 *L. icterohaemorrhagiae*-specific monoclonal antibody

2.2.12 Polyclonal *L. icterohaemorrhagiae* antiserum, rabbit origin

2.2.13 Goat anti-mouse IgG (H+L) horseradish peroxidase-labeled antibody (Jackson Immunoresearch, Inc., or equivalent)

2.2.14 Test bacterin(s) containing *L. icterohaemorrhagiae*

2.2.15 Reference bacterin containing *L. icterohaemorrhagiae* (must be approved by the Animal and Plant Health Inspection Service [APHIS])

2.2.16 Deionized or distilled water

3. Preparation for the test

3.1 Personnel qualifications/training

Technical personnel need a working knowledge of the use of general laboratory chemicals, equipment, and glassware; automated microplate washer and microplate reader; and data analysis software. They need specific training in the performance of this assay.

3.2 Preparation of equipment/instrumentation

Operate and maintain all equipment according to manufacturers' recommendations and applicable in-house standard operating procedures.

3.3 Preparation of reagents

3.3.1 Carbonate coating buffer--National Veterinary Services Laboratories (NVSL) Media #20034

Na_2CO_3	0.159 g
$NaHCO_3$	0.293 g
Deionized water	q.s. to 100 ml

Adjust pH to 9.6 ± 0.1. Store at 2°-7°C up to 1 wk.

3.3.2 Phosphate buffered saline (PBS)--NVSL Media #10559

NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.20 g
Deionized water	q.s. to 1 L

Adjust pH to 7.2 ± 0.1. Store at 20°-25°C no longer than 6 mo.

3.3.3 Polyvinyl alcohol, 1%, in PBS

PBS (see **Section 3.3.2**)	70 ml
Polyvinyl alcohol, 88% hydrolyzed, MW 13,000-23,000 (Aldrich Chemical, Cat. No. 36,317-0, or equivalent)	0.7 g

Stir to dissolve. Store at 20°-25°C no longer than 1 wk.

3.3.4 Phosphate buffered saline with 0.05% Tween 20 (PBS-Tween 20)--NVSL Media #30179

PBS (see **Section 3.3.2**)	1000 ml
Tween 20	0.50 ml

Store at 20°-25°C no longer than 6 mo.

3.3.5 Antibody diluent (quantity for 1 plate)

Polyvinyl alcohol, 1%, in PBS (see **Section 3.3.3**)	21.8 ml
Normal rabbit serum	200 µl

Mix immediately prior to use.

3.3.6 Phosphate buffer for antigen elution

KH_2PO_4 (Mallinckrodt, Inc., 7100, or equivalent)	8.2 g
Deionized water	94 ml

Adjust pH to 9.3 ± 0.1, or other appropriate pH as optimized for use with a specific bacterin. Store at 20°-25°C no longer than 1 mo.

3.3.7 Sodium desoxycholate elution buffer

Sodium desoxycholate (Difco Laboratories, 0248-13-7, or equivalent)	0.50 g
PBS (see **Section 3.3.2**)	100 ml

Store at 2°-7°C up to 30 days. Warm to 20°-25°C prior to use (buffer gels at 2°-7°C).

3.3.8 Monoclonal antibody (MAb) specific for *L. icterohaemorrhagiae*, clone 294-004

Obtain MAb 294-004 from the CVB-L. Store long-term at ≤-70°C. The MAb may be stored at 2°-7°C for several wk.

Note: MAb 294-004 does not recognize antigens in *L. icterohaemorrhagiae* serovar *bogvere* (type strain LT60-69); bacterins containing serovar *bogvere* must be assayed by an alternative procedure.

3.3.9 Polyclonal *L. icterohaemorrhagiae* antiserum, rabbit origin

Obtain the rabbit antiserum from the CVB-L. Store long-term at ≤70°C. The antiserum may be stored at 2°-7°C for several wk.

3.3.10 Bacterins containing *L. icterohaemorrhagiae* antigen

3.3.10.1 Reference bacterin

3.3.10.2 Test bacterin(s)

CRITICAL CONTROL POINT: Ideally, the reference and test bacterins should be produced by the same Outline of Production. If reference formulation differs from that of the test bacterin, the assay must be validated to show that this does not adversely affect assay performance or accuracy of results.

3.4 Preparation of the sample

Many bacterins do not require antigen-elution treatment prior to being serially diluted in twofold increments with PBS-Tween 20. Test representative batches of each adjuvanted product with and without each antigen-elution treatment to determine if the treatment specifically enhances antigen capture. If no enhancement of antigen capture can be demonstrated, test the bacterins without antigen-elution treatment. Treat the reference bacterin and the test bacterins by the same elution procedure. Alternative elution procedures, other than those described here, may be more appropriate for some bacterins.

3.4.1 Aluminum hydroxide-adjuvanted bacterins

Bacterins adjuvanted with aluminum hydroxide may be treated with either sodium citrate or phosphate buffer prior to making serial twofold dilutions in PBS-Tween 20.

3.4.1.1 Sodium citrate elution

Mix 1 g sodium citrate with 10 ml of bacterin (10% w/v). Place on an orbital shaker (100-120 rpm) overnight at 36°-38°C. Consider treated bacterin to be undiluted.

3.4.1.2 Phosphate buffer elution

Mix 1 ml of phosphate elution buffer with 1 ml of bacterin. Place on an orbital shaker (100-120 rpm) overnight at 36°-38°C. Consider treated bacterin to be diluted 1:2.

3.4.1.3 Oil-adjuvanted bacterins

Mix 1 ml of sodium desoxycholate elution buffer with 1 ml of bacterin. Place on an orbital shaker (100-120 rpm) overnight at 20°-25°C. Consider treated bacterin to be diluted 1:2.

4. Performance of the test

4.1 Dilute *L. icterohaemorrhagiae* rabbit antiserum to the current use dilution in cold carbonate coating buffer. Dispense 100 µl into each well of a 96-well microtitration plate (test plate). Seal plate and incubate at 2°-7°C for 16-20 hr. Coated plates may be stored at 2°-7°C up to 5 days.

4.2 Make twofold dilutions of reference and test bacterins, using PBS-Tween 20 as a diluent. Add 125 µl PBS-Tween 20 to each well of a clean microtitration plate (transfer plate). Place 125 µl of bacterin in the first well of each row. Test each bacterin in at least 3 replicate rows. Test the reference bacterin and the test bacterin on the same plate.

Use a multichannel pipetting device to make serial twofold dilutions of each bacterin across the plate (125 µl transfer volume). Reserve at least 2 unused wells on each plate to serve as blanks.

The use of at least 7 serial twofold dilutions per bacterin is recommended. Ideally, the selected bacterin dilutions should delineate the sigmoid curve from antigen saturation to antigen extinction for each bacterin. The dilutions used for the reference bacterin and the test bacterin may differ.

4.3 Wash the test plate once with 200 µl deionized or distilled H_2O (dH_20). Transfer 100 µl of liquid from each well of the transfer plate to the corresponding well on the test plate. Incubate the plates for 90 ± 5 min at 36°-38°C.

4.4 Wash the plates 3 times with dH_2O, 200 µl per wash.

4.5 Dilute MAb 294-004 to the current use dilution in antibody diluent, and add 100 µl to each well of the test plate. Incubate the plates for 60 ± 5 min at 36°-38°C.

4.6 Wash the plates 3 times with dH_2O, 200 µl per wash.

4.7 Dilute the goat anti-mouse IgG horseradish peroxidase conjugate to the current use dilution in antibody diluent. Add 100 µl to each well of the test plate. Incubate for 40 ± 5 min at 36°-38°C.

4.8 Wash the plates 3 times with dH_2O, 200 µl per wash.

4.9 Add ABTS substrate (100 µl) to all wells, and incubate plates for 15-30 min at 36°-38°C.

4.10 Read plates at 405/490 nm. Calculate the mean absorbance for the blank wells. Subtract the mean absorbance of the blank wells from each bacterin test well absorbance value prior to data analysis.

5. Interpretation of the test results

5.1 Relative potency calculation method

5.1.1 Use the current version of the *Relative Potency Calculation Software* (RelPot) to calculate the relative potency of the test bacterin as compared to that of the reference bacterin.

5.1.2 Do not use bacterin dilutions with mean absorbance values <0.050 (after subtraction of the mean absorbance of the blank) in the relative potency calculations.

5.1.3 Do not use regression lines with slopes >-0.100 in relative potency calculations. Enter a minimum slope (Min Slope) assay parameter of 0.100 in the spreadsheet in place of the 0.000 default.

5.1.4 Enter the reference and test bacterin data, and execute the relative potency program as outlined in the current version of MVSAM0318.

5.1.5 Report the highest relative potency (RP) value included in the top scores from each test as the RP for the test bacterin.

5.2 Requirements for a valid assay

5.2.1 An assay must meet the validity requirements in the current version of MVSAM0318 to be considered valid.

5.2.2 Lines determined by first-order linear regression of at least 3 contiguous points must have a correlation coefficient (r) of ≥0.95.

5.2.3 The reference regression line and the test bacterin regression line must show parallelism (slope ratio ≥0.80).

5.2.4 Assays that are not valid may be repeated up to a maximum of 3 times. If a valid assay cannot be achieved with 3 independent assays, the test bacterin is unsatisfactory.

5.3 Requirements for a satisfactory test bacterin

5.3.1 To be considered satisfactory, a test bacterin must have an RP value of ≥1.0. Test bacterins with RP values <1.0 on a valid assay may be retested by conducting 2 independent replicate tests in a manner identical to the initial test. If both retests are valid and the reported RP values of both of the retests are ≥1.0, the test bacterin is satisfactory.

6. Report of test results

Report the results of the test(s) as described in the current version of BBSOP0020.